

Indicators of tenderization are detectable by 12 h postmortem in ovine longissimus^{1,2}

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ABSTRACT: Postmortem changes in osmotic pressure; ionic strength; pH; temperature; μ - and m-calpain; calpastatin; desmin degradation; and myofibril fragmentation index (MFI) were determined in ovine longissimus muscle. Our objectives were to characterize changes in these variables and to identify postmortem time points at which significant proteolysis and tenderization (as measured by change in MFI) could be detected. Seven crossbred (Dorset \times Romanov) lambs were slaughtered, and samples of the longissimus muscle were removed at 0, 3, 6, 9, 12, 24, 72, and 360 h postmortem. Osmotic pressure increased ($P < 0.05$) from 379 to 528 mOsm during the postmortem storage period, with two-thirds of the increase occurring within the first 24 h. By measuring conductivity, we showed that ionic strength increased ($P < 0.05$) from 8.13 to 9.78 mS/cm during the storage period, which is equivalent to 79 and 97 mM NaCl solutions, respectively. In accordance with pH and temperature, conductivity reached ultimate levels at 24 h postmortem. Within 9 h postmortem, μ -calpain activity had decreased ($P < 0.05$) from at-

death values and continued to decrease until 72 h, at which time it was undetectable. It was still possible to detect the 76-kDa isoforms (a product of the autolysis of the 80-kDa subunit of μ -calpain) immunologically, which implies that the loss of activity was not caused by extensive autolysis. In contrast, m-calpain activity remained constant throughout the aging period, whereas calpastatin activity was stable until 24 h postmortem, after which it gradually decreased. Autolysis products of μ -calpain were detected at 3 h postmortem, indicating that μ -calpain was activated some time between 0 and 3 h postmortem. Moreover, the effect of μ -calpain activity on myofibrillar substrates was first observed at 9 h postmortem, when a 23% loss of native desmin was detected. This degradation translated into an increase in MFI at 12 h. Collectively, these results imply that μ -calpain is active in postmortem muscle in the presence of calpastatin, and that effects of μ -calpain activity as determined by increased MFI are detectable during the first 12 h postmortem.

Key Words: Calpains, Conductivity, Osmolality, Ovine, Proteolysis

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Introduction

Tenderness is the single most important factor influencing consumer acceptance of meat. During the first 24 h postmortem, the LM goes through a toughening phase due to sarcomere shortening during rigor devel-

opment (Wheeler and Koohmaraie, 1994). The subsequent tenderization process is far more variable than the rigor toughening process. Evidence shows that the calpain proteolytic system is responsible for this tenderization process (for reviews, see Goll et al., 1991; Koohmaraie, 1992c). μ -Calpain degrades key myofibrillar and associated proteins, leading to weakening of the myofibrils and tenderization. Variation in the extent of postmortem proteolysis by μ -calpain accounts for more of the variation in tenderness of LM after postmortem storage than any other factor.

To prevent tough meat from reaching consumers, the U.S. meat industry has expressed the desire for a tenderness-based classification system to sort carcasses based on tenderness after postmortem storage. However, prediction of tenderness is difficult owing to large animal variation in the rate of tenderization. A tenderness-based beef classification system will very likely be

¹Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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used between 48 and 72 h postmortem. Therefore, the accuracy of a prediction system would be optimized if the tenderization process could be accelerated. To be able to accelerate the tenderization process, it is essential to understand changes taking place during early-postmortem storage of meat and how these changes affect the calpain proteolytic system. Thus, the objectives of this study were to characterize early-postmortem changes in multiple physiochemical traits and calpain and calpastatin activities and to identify the earliest time points at which significant changes in proteolysis and tenderization could be detected.

Materials and Methods

Animals

The Roman L. Hruska U.S. Meat Animal Research Center (MARC) Animal Care and Use Committee approved the use of animals in this study. Seven crossbred (Dorset \times Romanov) lambs were slaughtered, and carcasses were stored at 1°C during the postmortem storage period. Portions of the LM were removed at 0, 3, 6, 9, 12, 24, 72, and 360 h postmortem. At each sampling time, a 5-cm section was removed alternating between LM on the left and right sides of the carcass for each sampling time (left side sampled at 0, 6, 12, and 72 h; right side sampled at 3, 9, 24, and 360 h). Because the dissection of a sample during the first 24 h could introduce artifacts on the subsequent sample (i.e., owing to altered temperature decline or by inducing muscle shortening), 5-cm gaps were left between the 0- and 6-h samples and the 3- and 9-h samples. A 2.54-cm gap was left between the 6- and 12-h samples and the 9- and 24-h samples, but no gap was left between 12 and 72, or between 24 and 360 h, because rigor was completed by 12 h postmortem. The portions of the LM were trimmed of visible fat and connective tissue, diced into small pieces, and divided among the analyses.

Temperature and pH

The temperature of LM left on the carcass during the aging period was measured using an RD-Temp temperature data logger (Omega, Stamford, CT). The pH was determined by homogenizing 1 g of muscle in 10 mL of pH buffer (5 mM iodoacetate, 150 mM KCl) according to Bendall (1973). The pH of the suspension was measured using a PHM82 standard pH meter (Radiometer, Copenhagen, Denmark).

Osmotic Pressure and Ionic Strength

For measurements of osmotic pressure and ionic strength, 5 g of muscle was macerated in a coffee grinder using five 1-s bursts. The macerated sample was centrifuged at $37,500 \times g$ (maximum) for 2 h at 4°C. Supernatant was collected, and measurements were performed after this sample had reached room

temperature. Osmotic pressure was determined using the Advanced Micro-Osmometer (Model 3MO Plus; Advanced Instruments, Inc., Norwood, MA), and ionic strength was determined using a CDM210 conductivity meter (Radiometer).

Calpains and Calpastatin

Enzymatic activity of the calpain system was determined using four methods designed to give individual activities of μ -calpain, m-calpain, and calpastatin, as well as net proteolytic activities. Whole muscle extracts were prepared by homogenizing 45 g of muscle in either 3 vol of prerigor extraction buffer (50 mM Tris base, 10 mM EDTA, 0.05% 2-mercaptoethanol [MCE], 2 mM phenylmethylsulfonyl fluoride [PMSF], 100 mg/L ovomucoid, 16 mg/L leupeptin, adjusted with HCl to pH 8.3) or 3 vol of postrigor extraction buffer (100 mM Tris base, 10 mM EDTA, 0.05% MCE, 2 mM PMSF, 100 mg/L ovomucoid, 16 mg/L leupeptin, pH 8.3) using a Waring blender (Dynamics Co. of America; New Hartford, CT) set at high speed, with three 30-s bursts interspersed with 30-s cooling periods. The switch from prerigor extraction buffer to postrigor extraction buffer was made once the pH of the muscle had declined below 6.2 (Veiseth and Koohmaraie, 2001). Supernatant was collected after centrifugation at $16,000 \times g$ for 2 h, and, to decrease the conductivity to that of DEAE-Sephacel equilibrating buffer, the supernatant was then dialyzed against dialysis buffer (40 mM Tris base, 5 mM EDTA, 0.05% MCE, pH 7.35) overnight at 4°C. The dialyzed extract was clarified by centrifugation at $28,000 \times g$ for 1 h and filtered through glass wool. This extract was used to determine the components and net calpain activity using four methods. To prepare the crude sample for estimation of its proteolytic activity, 5 mL of the extract was dialyzed against elution buffer (40 mM Tris base, 0.5 mM EDTA, 0.05% MCE, pH 7.35) overnight. This dialysis step reduced the EDTA concentration from 5 to 0.5 mM. After dialysis, the caseinolytic activity of the crude extract was determined using [14 C]casein as described by Koohmaraie (1992b). Briefly, the reaction consisted of 100 μ L of crude sample, 200 μ L of elution buffer, 20 μ L of 100 mM CaCl_2 , and 25 μ L of [14 C]casein. After incubating for 1 h at 25°C, the reaction was stopped by adding 100 μ L of 10 mg/mL BSA and 500 μ L of ice-cold 10% trichloroacetic acid (TCA). The reactions were centrifuged at $8,800 \times g$ for 20 min, and an aliquot of the TCA-soluble fraction (475 μ L) was mixed with 5 mL of scintillation fluid. Radioactivity was measured using a Packard liquid scintillation counter (model 460; Packard Instruments, Meriden, CT). To account for caseinolytic activity in the crude extract caused by substances other than calpain, for each sample we included a control, in which 100 μ L of elution buffer was replaced by 100 μ L of semipurified calpastatin. Calpain proteolytic activity in the crude extract was calculated as the difference between assays with and without semipurified calpastatin.

The remaining muscle extract was divided into three equal parts and gravity-loaded onto three 35-mL chromatography columns packed with DEAE-Sephacel (Pharmacia LKB Biotechnology, Uppsala, Sweden). Columns were washed using elution buffer until the absorbance at 278 nm of the outflow was less than 0.1. Three strategies were used to elute proteins bound to the columns. Proteins bound to the first column were separated using a continuous gradient of from 25 to 400 mM NaCl in elution buffer (henceforth referred to as *gradient elution*). The μ -calpain, m-calpain, and calpastatin activities were determined as described by Koohmaraie (1990). Proteins bound to the second DEAE-Sephacel column were eluted in two steps of 200 and 400 mM NaCl in elution buffer (referred to as *two-step elution*). In the two-step method, the 200 mM elution included both calpastatin and μ -calpain. Assaying for calpain activity estimated the μ -calpain activity in the presence of calpastatin. A one-step elution of 400 mM NaCl in elution buffer was used to elute proteins bound to the third column (referred to as *one-step elution*). In the one-step elution, the 400 mM elution included all calpain components (μ -calpain, m-calpain, and calpastatin). Thus, assaying for calpain activity estimates the combined μ -calpain and m-calpain activities in the presence of calpastatin. All elutions were performed using a flow rate of 30 mL/h, and 140 fractions (3 mL) or 20 fractions (5 mL) were collected from the gradient and stepwise elutions, respectively. Fractions collected during the second step (400 mM NaCl) of the two-step elution were assayed for m-calpain activity as described by Koohmaraie (1990). Fractions from the one-step elution and the first step (200 mM NaCl) of the two-step elution were assayed for proteolytic activity according to the [14 C]casein assay described for crude samples with small modifications. Briefly, these reactions consisted of 25 μ L of sample, 125 μ L of elution buffer, 10 μ L of 100 mM CaCl_2 , and 25 μ L of [14 C]casein. After incubations, 100 μ L of 10 mg/mL BSA and 300 μ L of ice-cold 10% TCA were added. After centrifugation, 295 μ L of the supernatant was mixed with 5 mL of scintillation fluid before measuring its radioactivity.

Myofibril Fragmentation Index

The myofibril fragmentation index (**MFI**) was determined on two 4-g muscle samples following the procedure of Olson et al. (1976) as modified by Culler et al. (1978).

Immunoblotting

Samples for immunoblotting analysis were snap-frozen in liquid N_2 at the time of sampling and stored at -80°C . For sample extraction, 300 mg of frozen muscle was homogenized in 0.9 mL of postrigor extraction buffer using a Polytron (Brinkman Instruments, Westbury, NY) set on speed setting 6 with three bursts of 15 s interspersed with 15-s cooling periods. After cen-

trifugation at $8,800 \times g$ for 30 min, the supernatant (containing soluble proteins) was collected and retained, whereas the pellet (containing insoluble proteins) was resuspended in 0.6 mL of 100 mM NaCl with 1 mM NaN_3 using a Polytron. The resuspended pellet was centrifuged at $8,800 \times g$ for 5 min, and the supernatant was discarded. The pellet was once again resuspended and centrifuged, before being resuspended for a final time in 0.9 mL of 100 mM NaCl with 1 mM NaN_3 . Protein concentrations of the resuspended pellet and the original supernatant were determined with a Coomassie-based protein assay (Bio-Rad, Hercules, CA). Samples were diluted to a final protein concentration of 3 mg/mL with protein denaturing buffer (125 mM Tris base, 4% SDS, 0.5% MCE, 0.04% bromophenol blue, 20% glycerol [wt/vol], pH 6.8) and heated for 20 min at 50°C to ensure denaturation. The SDS-PAGE was performed according to Laemmli (1970) using 10% separating gels (37.5:1 ratio of acrylamide to bis N,N'-methylene-bis-acrylamide) with 4% stacking gels (37.5:1). For analysis of desmin, 9 μ g of the pellet fraction was electrophoresed at 200 V for 45 min before being transferred onto Hybond-P polyvinylidene fluoride (PVDF) membranes (Amersham Biosciences, Uppsala, Sweden) for 2.5 h at 200 mA. For analysis of μ -calpain, 36 μ g of the supernatant fraction was loaded and gels were run at 200 V for 135 min and transferred to PVDF membranes at 300 mA for 1 h. All membranes were blocked with 2.5% (vol/vol) sheep serum in Tris-buffered saline (20 mM Tris base, 137 mM NaCl, 5 mM KCl, pH 7.4) containing 0.05% Tween-20 (TTBS) for 1 h. Primary and secondary antibodies were diluted in TTBS and incubated for 1 h at room temperature with gentle shaking, and membranes were washed three times (5 min per washing) with TTBS after both incubations. The primary antibodies were mouse anti-desmin, used at a 1:40 dilution (D3; Danto and Fischman, 1984) and mouse anti- μ -calpain, used at a 1:25 dilution (B2F9; Geesink and Koohmaraie, 1999b), whereas the secondary antibody was Immunopure goat anti-mouse IgG horseradish peroxidase conjugate (Pierce, Rockford, IL), used at 1:25,000 and 1:15,000 dilutions for desmin and μ -calpain, respectively. Antibody binding was visualized by incubating the membranes with SuperSignal Chemiluminescence substrate (Pierce), and images were captured with a ChemiImager 5500 digital imaging analysis system (Alpha Innotech, San Leandro, CA). Protein bands were quantified using the ChemiImager 5500 digital imaging analysis system, and the extent of desmin degradation was expressed as the percentage loss of density of the desmin band at different sampling times compared to at-death levels from the same animal.

Statistical Analysis

Data were analyzed by using the GLM procedures of SAS (SAS Inst., Inc., Cary, NC) for a repeated measures design. The model included the main effect of time (0,

Table 1. Postmortem changes in physiochemical traits in ovine longissimus muscle

Postmortem sampling time, h	Temperature, °C	pH	Osmolality, mOsm	Conductivity, mS/cm	
				23°C	Adjusted for muscle temp.
0	41.2 ^a	6.85 ^a	379 ^e	8.13 ^d	11.30 ^a
3	22.8 ^b	6.38 ^b	420 ^d	9.13 ^c	9.13 ^b
6	11.5 ^c	6.28 ^b	441 ^{cd}	9.11 ^c	7.11 ^c
9	5.1 ^d	6.12 ^c	459 ^c	9.43 ^{bc}	6.08 ^d
12	3.6 ^e	5.99 ^c	459 ^c	9.21 ^c	5.71 ^e
24	1.5 ^f	5.62 ^d	481 ^b	9.59 ^{ab}	5.75 ^{de}
72	1.6 ^f	5.62 ^d	500 ^b	9.70 ^{ab}	5.82 ^{de}
360	1.8 ^f	5.66 ^d	528 ^a	9.78 ^a	5.87 ^{de}
SEM	0.39	0.05	7.78	0.13	0.14

^{a,b,c,d,e,f}Within a column, means without a common superscript letter differ ($P < 0.05$).

3, 6, 9, 12, 24, 72, and 360 h). When the main effect was significant ($P < 0.05$), least squares means separation was accomplished by the PDIFF option (a pairwise t -test).

Results

Physiochemical Traits. As expected, the temperature of LM decreased ($P < 0.05$) from 41.2°C at slaughter to 1.5°C at 24 h postmortem (Table 1). Muscle pH decreased ($P < 0.05$) from an initial value of 6.85 to 5.62 at 24 h postmortem. Osmotic pressure increased ($P < 0.05$) from an at-death level of 379 to 528 mOsm at 360 h postmortem, with one-third of the increase occurring after 24 h (Table 1). Conductivity of muscle juice (measured at 23°C) increased ($P < 0.05$) from 8.13 mS/cm to 9.59 mS/cm during the first 24 h postmortem. Subsequent increases were not significant ($P > 0.05$), and at 360 h postmortem conductivity was stabilized at 9.78 mS/cm. The greatest single increase ($P < 0.05$) occurred between 0 and 3 h postmortem and accounted for 60% of the overall increase during the storage period (Table 1). Because conductivity measurements are highly affected by temperature, a standard curve was prepared to establish the relationship between these two variables. Using this standard curve, the muscle temperature profile and the conductivities measured at room temperature, the conductivity of in vivo muscle juice was estimated. These temperature-adjusted conductivities declined ($P < 0.05$) during the first 12 h of postmortem storage from 11.3 to 5.7 mS/cm, which is strikingly different from the conductivities measured at 23°C (Table 1). Thus, pH, temperature, and conductivity seem to reach ultimate levels at 24 h postmortem, whereas the osmotic pressure continues to increase with further storage.

Calpain Proteolytic Activities. As early as 9 h postmortem, measurable μ -calpain activity decreased ($P < 0.05$) relative to at-death levels (Table 2; gradient elution). This decline ($P < 0.05$) continued between 9 and 72 h, and, by 72 and 360 h postmortem, μ -calpain activity was no longer detected using the standard caseinolytic assay. Calpastatin activity also decreased with post-

mortem storage (Table 2; gradient elution). Although no ($P > 0.05$) changes were detected during the first 12 h, calpastatin activity at 24 h was lower ($P < 0.05$) than at death, and further declines ($P < 0.05$) were measured at 72 and 360 h. With respect to m-calpain activity, the two different elution methods generated different results (Table 2). Using the gradient elution, m-calpain activity was found to decrease ($P < 0.05$) slightly with storage; however, the two-step elution showed that m-calpain activity did not ($P > 0.05$) decrease with postmortem storage. The first step (200 mM NaCl) of the two-step elution coeluted calpastatin and μ -calpain, whereas the one-step elution, using 400 mM NaCl, resulted in coelution of calpastatin and μ - and m-calpain. These eluents and the crude extracts were analyzed to discover whether calpain activity could be detected in the presence of calpastatin (Table 2). Coelution of calpastatin and μ -calpain revealed that a small amount of proteolytic activity was detectable at all time points postmortem (Table 2; two-step elution). Not surprisingly, therefore, coelution of calpastatin and both μ - and m-calpain resulted in higher net proteolytic activities (Table 2; one-step elution and crude extract). Using the one-step elution, the net proteolytic activity was unchanged during the first 24 h of postmortem storage, but increased ($P < 0.05$) at both 72 and 360 h. Crude extracts showed a similar trend, with an increase ($P < 0.05$) at 360 h postmortem. The correlation of the ratio of μ - plus m-calpain-to-calpastatin activities with either one-step elution or crude extract activities was 0.95 (data not shown). Crude extracts assayed with semipurified calpastatin showed no detectable ($P > 0.05$) activities, indicating that there was no non-calpain proteolytic activity in the crude extracts at any time postmortem (data not shown).

Postmortem Proteolysis. The MFI increased with storage and, relative to at-death values, an increase ($P < 0.05$) in MFI was detected as early as 12 h postmortem (Table 2). Further increases ($P < 0.05$) in MFI were detected at all sample times from 12 to 360 h postmortem. Degradation of desmin paralleled the MFI changes, and desmin degradation as early as 9 h was greater than at death ($P < 0.05$). Further increases in

Table 2. Postmortem changes in activities of the calpain proteolytic system, myofibril fragmentation index (MFI) and desmin degradation in ovine longissimus muscle

Postmortem sampling time, h	Gradient elution ^a			Two-step elution		One-step elution ^b	Crude extract ^b	MFI	Desmin ^c
	μ -Calpain	m-Calpain	Calpastatin	μ -Calpain and calpastatin ^b	m-Calpain ^a				
0	1.29 ^d	1.15 ^d	3.08 ^d	69.7 ^{de}	1.43 ^d	138.7 ^f	140.5 ^{ef}	36.7 ⁱ	0 ⁱ
3	1.26 ^d	1.03 ^{de}	2.39 ^{de}	71.3 ^{de}	1.14 ^{de}	209.3 ^{ef}	224.0 ^{ef}	39.3 ^{hi}	9 ^{hi}
6	1.29 ^d	0.99 ^{def}	2.70 ^d	73.5 ^d	1.28 ^{de}	273.8 ^{ef}	244.0 ^{ef}	42.6 ^{gh}	11 ^{ghi}
9	0.86 ^e	0.89 ^{efg}	2.69 ^d	42.0 ^{de}	1.11 ^e	143.8 ^f	98.5 ^f	41.2 ^{hi}	23 ^{figh}
12	0.74 ^e	0.85 ^{efg}	2.60 ^{de}	39.0 ^e	1.09 ^e	140.8 ^f	79.0 ^f	45.9 ^g	24 ^{fg}
24	0.35 ^f	0.76 ^g	1.98 ^e	60.7 ^{de}	1.06 ^e	245.8 ^{ef}	95.5 ^f	56.0 ^f	31 ^f
72	ND ^g	0.81 ^{fg}	0.97 ^f	42.8 ^{de}	1.18 ^{de}	394.7 ^e	308.5 ^e	79.8 ^e	62 ^e
360	ND ^g	0.87 ^{efg}	0.13 ^g	63.0 ^{de}	1.24 ^{de}	927.8 ^d	792.7 ^d	88.7 ^d	94 ^d
SEM	0.07	0.07	0.25	11.75	0.11	73.45	64.66	1.62	5.32

^aStandard casein assay (activity given as units/g of muscle).

^b[¹⁴C]casein assay (activity given as μ g casein degraded/g muscle).

^cLoss of desmin from at-death levels, %.

^{d,e,f,g,h,i}Within a column, means without a common superscript letter differ ($P < 0.05$).

ND = not detectable.

desmin degradation occurred with a 62% loss at 72 h and a 94% loss at 360 h postmortem (Table 2). Autolysis of the large subunit of μ -calpain was first detected at 3 h postmortem, where both the 78- and the 76-kDa autolysis products were observed (Figure 1). There was a gradual decline in the intensity of the 80-kDa band until 72 h postmortem, after which it was no longer detectable. The intensity of the 76-kDa band, however, increased throughout the postmortem storage period.

Discussion

One of the objectives of this study was to characterize changes in physiochemical traits during postmortem storage of meat. The decline in temperature and pH during the first 24 h postmortem corresponds well with earlier results reported for lamb LM (Koohmaraie et al., 1991). We used conductivity to estimate ionic strength. Ionic strength is defined by an equation and to calculate ionic strength, the concentration and number of charge ions in the solution must be known (Segel, 1976). Ionic strength (conductivity) and osmotic pressure (osmolality) are both related to solute concentration; conduc-

tivity only measures solutes with a net charge, whereas osmolality is a measure of total solutes. Thus, the increase in 23°C conductivity with postmortem storage reflects the increasing concentration of solutes with a net charge (and parallels increases in total solute concentration [osmolality] through 24 h). However, temperature-adjusted conductivity reflects the decline in the activity, or ionic strength, of those charged solutes due to the decline in muscle temperature during postmortem storage.

The reported change in osmotic pressure, increasing from an at-death level of 379 to 528 mOsm at 360 h postmortem, agrees with previous studies. Specifically, Winger and Pope (1981) found that beef muscle had an osmotic pressure of 300 mOsm prerigor, which increased to 480 to 540 mOsm postrigor. Unlike pH and temperature, changes in osmotic pressure did not stop at 24 h and continued throughout the storage period. Previously, Ouali (1990, 1992) found that the correlation between pH decline and the postmortem increase in osmotic pressure was 0.97 and that ultimate osmolality in bovine muscle was reached at 24 h (Ouali et al., 1991). Our results show that, although changes in osmotic pressure were inverse to the decrease in pH during the first 24 h postmortem, there was a further increase in osmotic pressure between 24 h and 360 h, which accounted for one-third of the overall increase in osmotic pressure. This discrepancy between our results and the results presented by Ouali et al. (1991) may be an effect of species and/or use of different postmortem temperature regimes (1 vs. 15°C until rigor completion and 4°C thereafter).

To the best of our knowledge, this paper is the first to report a measure of conductivity of postmortem muscle. Previously, measurements of osmotic pressure have been used to calculate the ionic strength of postmortem muscle. This approach may have been inspired by Winger and Pope (1981), who indicated that the osmotic

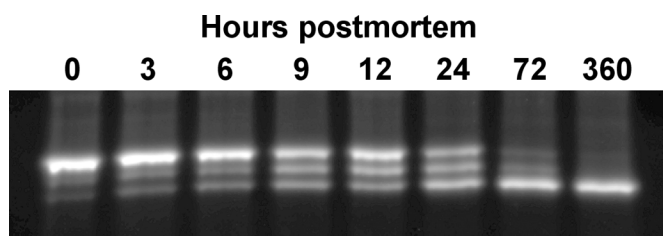


Figure 1. Western blot analysis of the large subunit (80 kDa) of μ -calpain from the soluble fraction during postmortem storage of ovine longissimus. The molecular masses of the top, middle, and bottom bands are 80, 78, and 76 kDa, respectively.

pressure of postrigor muscle (480 to 540 mOsm) was equivalent, or iso-osmotic, to a 275 to 295 mM NaCl solution, whereas prerigor muscle was iso-osmotic to a 165 mM NaCl solution. This statement was misinterpreted to mean that the ionic strength of postrigor muscle is comparable to these salt solutions (Wu and Smith, 1987; Ouali, 1990, 1992; Geesink and Koohmaraie, 2000). Although Winger and Pope (1981) warned against the use of salt solutions for *in vitro* studies of postrigor muscle events, many experiments have been performed using salt solutions with osmotic pressures equivalent to postmortem muscle. It has been shown that ionic strengths of 240 to 300 mM NaCl are high enough to weaken myofibrils, making them more susceptible to proteolysis (Wu and Smith, 1987) and that activities of calpains are affected by elevated ionic strengths (Kendall et al., 1993; Geesink and Koohmaraie, 2000). Our results showed that the 23°C conductivity (which is a direct measure of ionic strength) of ovine LM increased from 8.13 to 9.78 mS/cm during postmortem storage, which is equivalent to 79 and 97 mM NaCl solutions, respectively. As with osmotic pressure, the changes in 23°C conductivity were inversely related to the decrease in pH, reaching ultimate values at 24 h postmortem. The largest single increase in 23°C conductivity occurred between 0 and 3 h postmortem, with an increase from 8.13 to 9.13 mS/cm (79 to 90 mM NaCl). Thus, for most of the postmortem storage period, the ionic strength of the muscle was equivalent to NaCl solutions of 90 to 97 mM. Consequently, results from studies using salt solutions higher than 100 mM NaCl are not representative of postmortem muscle and their conclusions may be unfounded. There is a strong relationship between temperature and conductivity, which is emphasized by comparing the conductivity observed at 23°C with the conductivity adjusted for muscle temperature. To accurately assess the changes in concentration of charged solutes from a sample, the effect of temperature should be removed; all samples should be measured at consistent temperature (e.g., 23°C). However, to accurately assess the activity, or ionic strength, of those solutes *in vivo*, the temperature-adjusted conductivity should be used. The *in vivo* conductivity declined as temperature declined; thus, the effective ionic strength for most of the postmortem period was equivalent to 53 to 90 mM NaCl solutions, which is even lower than estimated from the 23°C conductivity above. Our results showed an increase in charged solutes during the first 24 h postmortem but a decline in ionic strength. This leads us to conclude that the effect of ionic strength on postmortem myofibrils and calpain proteolysis would be far less than previously estimated.

The calpain proteolytic system is known to be responsible for the postmortem tenderization of meat, and numerous studies have been performed to determine the activity of μ -calpain, m-calpain and their inhibitor calpastatin during postmortem storage (for reviews, see Goll et al., 1991; Koohmaraie, 1992c). Both calpain proteases require Ca^{2+} to be proteolytically active, and, in

the presence of sufficient Ca^{2+} , will undergo autolysis. *In vitro*, the Ca^{2+} -requirement for autolysis is higher than that required for proteolysis (Cong et al., 1989). Consequently, evidence of *in vivo* autolysis is indicative of the Ca^{2+} concentration being sufficient for proteolytic activity. In general, reports show m-calpain activity is unchanged during postmortem storage of meat (Vidalenc et al., 1983; Ducastaing et al., 1985; Koohmaraie et al., 1987); however, some groups have reported a decline in m-calpain (Sensky et al., 1996, 1999; Beltran et al., 1997). We have previously shown that this discrepancy most likely results from the use of inappropriate extraction buffers (Veiseth and Koohmaraie, 2001). Using casein zymography, Veiseth et al. (2001) showed that m-calpain does not undergo autolysis, and is, therefore, not active under postmortem conditions. However, in the present study, the gradient elution of m-calpain demonstrated a 25% decrease in activity during the storage period (gradient elution; Table 2). This decrease was contradicted by the results from the two-step elution, which gave no evidence of a change in m-calpain activity during the postmortem storage. Based on the available evidence, we have concluded that m-calpain is not active postmortem and that any decrease in m-calpain activity detected using a gradient elution is an artifact introduced by the elution profile. For example, the peak of m-calpain activity might become broader and shallower, causing the rejection of low activity-containing fractions, and, thus, lowering the estimated activity.

Several studies have demonstrated a negative relationship between calpastatin activity and proteolysis in postmortem muscle. Using myofibril incubations, Geesink and Koohmaraie (1999a) showed that both the rate and extent of μ -calpain-mediated proteolysis decreased with increasing calpastatin activities. *In vivo*, the rate of desmin degradation in postmortem bovine, ovine, and porcine LM seems to correspond to their respective at-death levels of calpastatin (Koohmaraie et al., 1991). In contrast to m-calpain levels, which remain constant, calpastatin levels are known to decrease during postmortem storage. In bovine muscle, Boehm et al. (1998) found that calpastatin activities decreased to 60 and 30% of at-death levels at 1 and 7 d postmortem, respectively. Koohmaraie et al. (1995) reported a similar trend in lambs, showing a 62.5% decline over 7 d. More recently, Geesink and Koohmaraie (1999b) demonstrated that the elevated level of calpastatin in callipyge lambs resulted in both decreased rate and extent of postmortem proteolysis. In agreement with previous studies, our results show a decrease in calpastatin levels during postmortem storage. The degradation of calpastatin by μ -calpain has been implicated as the mechanism responsible for loss of calpastatin activity during postmortem storage of meat (Doumit and Koohmaraie, 1999).

The existence of two calpain proteases (μ - and m-calpain) has prompted researchers to investigate which of the two is predominantly responsible for tenderiza-

tion of meat. Koohmaraie et al. (1987) suggested that μ -calpain alone was responsible for tenderization, and this assertion was recently confirmed by Veiseth et al. (2001), when they showed that only μ -calpain is active in postmortem muscle. In the present experiment, μ -calpain autolysis was first detected at 3 h postmortem. Activity of μ -calpain did not decrease until 9 h postmortem, which suggests that the 78- and 76-kDa autolysis products of μ -calpain were active under postmortem conditions between 3 and 9 h. Although our assay method was unable to detect μ -calpain activity at 72 and 360 h postmortem, Geesink and Koohmaraie (1999b) demonstrated that μ -calpain activity could be detected at 56 d postmortem in ovine LM when using the more-sensitive [14 C]casein assay. In vitro, the decrease in μ -calpain activity is a consequence of extensive autolysis. Studies performed by Koohmaraie (1992a) showed that complete autolysis of the 80-kDa subunit of μ -calpain resulted in fragments ranging from 61 to 23 kDa, and only the 61-kDa fragment retained proteolytic activity. The observation that a small amount of residual calpain activity is detected even after extensive autolysis is explained by experiments performed by Cottin et al. (2001), who showed a decrease in μ -calpain autolysis both when substrate (casein) was present and when the concentration of μ -calpain was reduced. Cottin et al. (2001) concluded that autolysis of μ -calpain was an intermolecular process. As a consequence, theoretically, there will always be a residual amount of μ -calpain activity in postmortem muscle regardless of storage time. With respect to protein product of the 80-kDa autolysis, our Western blots showed 78- and 76-kDa fragments as early as 3 h postmortem. The 78-kDa fragment was detectable until 72 h postmortem, whereas the 76-kDa fragment increased in amount throughout storage. Because the 76-kDa fragment is known to be proteolytically active in vitro, its abundance at 360 h would seem to be inconsistent with the activity at this time. Geesink and Koohmaraie (2000) addressed this discrepancy and showed that autolyzed μ -calpain is unstable at high ionic strengths. They concluded that the decline in μ -calpain activity in postmortem muscle is a result of instability of the partially autolyzed μ -calpain. This instability may result from exposure of hydrophobic regions, following autolysis, leading to aggregation of partially autolyzed large subunits and the formation of inactive dimers (D. E. Goll, personal communication).

A concern that this research sought to address was whether μ -calpain could be active in postmortem muscle and, therefore, be responsible for postmortem tenderization. Two arguments have been used against this role for μ -calpain. First, doubts have been raised about whether μ -calpain is active at the typical pH and temperature of postrigor muscle (pH 5.5 and 5°C). This was addressed by Koohmaraie et al. (1986), who showed that μ -calpain retained 24 to 28% of its maximum activity (pH 7.5 and 25°C) under conditions simulating postmortem muscle. In addition, Koohmaraie et al. (1986)

showed that this level of activity was sufficient to reproduce most of the changes found in postmortem muscle. The results of Koohmaraie et al. (1986) were later confirmed by Huff-Lonergan et al. (1996). The second argument against the role of μ -calpain is the excess of calpastatin in muscle. A typical ratio of μ -calpain to calpastatin in lamb LM at death is 1:3.5 units per gram of muscle (Koohmaraie et al., 1995). Myofibril incubations with μ -calpain and calpastatin have shown that calpastatin limits both the rate and extent of proteolysis and autolysis of μ -calpain and that, even at a 1:4 ratio of μ -calpain to calpastatin, μ -calpain activity is not completely inhibited (Geesink and Koohmaraie, 1999a). Our assays of coeluted μ -calpain and calpastatin support the conclusion of Geesink and Koohmaraie (1999a). As expected, the coelution of calpain and calpastatin using the one-step elution and the crude extract gave similar results, and, in both cases, net positive proteolytic activity was detected. Although there were no significant changes in net proteolytic activity during the first 24 h postmortem, the decrease in μ -calpain activity preceded the decrease in calpastatin activity, causing a slight reduction in net activity at 9 and 12 h postmortem in both of the coelution approaches and in the crude extract. However, at 72 and 360 h postmortem there was an increase in activity in the one-step elution and the crude extract, which is explained by a decrease of calpastatin activity, the absence of μ -calpain, and stable m-calpain activity. The fact that we detected proteolytic activity from coelution of μ -calpain and calpastatin at 72 and 360 h postmortem implies that μ -calpain is still active in the tissue at those times. Thus, the lack of μ -calpain activity detected with our standard assay at late postmortem times (gradient elution; Table 2) was clearly a sensitivity limitation of the method.

Having determined that μ -calpain is active in the presence of calpastatin and that its activity is detectable at 3 h postmortem, we sought to determine how early this activity would express itself in terms of the proteolysis of myofibrillar proteins. Previously, Wheeler and Koohmaraie (1994) were able to detect evidence of proteolytic degradation at 12 h postmortem in ovine longissimus and were able to confirm desmin and troponin-T degradation at 24 h. Our results show significant desmin degradation as early as 9 h postmortem, which continued until 360 h, when 94% of at-death levels of desmin was degraded. It is known that sensory panel tenderness is related to both Warner-Bratzler shear force and MFI in bovine LM (Whipple et al., 1990; Crouse et al., 1991); however, owing to a limited amount of sample, we chose to measure MFI only as an index of tenderness. Consistent with Koohmaraie et al. (1987) using bovine LM, we observed an increase in MFI at 12 h postmortem, with further improvements throughout the storage period. It appears that the increase in MFI is a consequence of μ -calpain proteolytic activity. As mentioned previously, the early-postmortem period represents a time of both toughening, caused by sarcomere shortening during rigor development, and tender-

ization, caused by proteolysis. Evidence of proteolysis and improvements in MFI at 9 and 12 h, respectively, suggest that the tenderization process has been initiated.

Implications

Results of the present study indicate that various levels of μ -calpain activity can be detected in the presence of calpastatin levels previously shown to be sufficient to completely inhibit μ -calpain *in vitro*. Additionally, evidence of μ -calpain activation shortly after slaughter was demonstrated by the degradation of desmin at 9 h postmortem and an increased myofibril fragmentation index (indicative of improvement in tenderness) by 12 h. These results imply that significant tenderization occurs during the first 24 h postmortem (37% of the total changes in myofibril fragmentation index) and that efforts should be directed to devise methods of accelerating tenderization during the first 24 h. Such technology would be of great value to the meat industry. Furthermore, the characterization of postmortem changes in multiple physiochemical traits can be used as a baseline for more detailed analysis of the calpain proteolytic system, which should assist in design of experiments aiming to accelerate and enhance the tenderization process.

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